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## **Penetration depth of corneal cross-linking with riboflavin and UV-A (CXL) in horses and rabbits**

Gallhoefer, Nicolin S ; Spiess, Bernhard M ; Guscetti, Franco ; Hilbe, Monika ; Hartnack, Sonja ;  
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# Penetration depth of corneal cross-linking with riboflavin and UV-A (CXL) in horses and rabbits

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## Abstract

**Objective** CXL penetration depth is an important variable influencing clinical treatment effect and safety. The purposes of this study were to determine the penetration depth of CXL in rabbit and equine corneas in epithelium-on and epithelium-off procedures and to assess an *ex vivo* fluorescent biomarker staining assay for objective assessment of CXL penetration depth.

**Procedures** CXL treatment was performed according to a standardized protocol on 21 and 17 rabbit eyes and on 12 and 10 equine eyes with and without debridement, respectively. Control corneas were treated similarly, but not exposed to CXL. Hemicorneas were stained with either phalloidin and DAPI to visualize intracellular F-actin and nuclei, or with hematoxylin and eosin. Loss of actin staining was measured and compared between groups.

**Results** Epithelium-off CXL caused a median actin cytoskeleton loss with a demarcation at 274 µm in rabbits and 173 µm in horses. In non-CXL-treated controls, we observed a median actin cytoskeleton loss with a demarcation at 134 µm in rabbits and 149 µm in horses. No effect was detected in the epithelium-on procedure.

**Conclusions** CXL penetration depth, as determined by a novel *ex vivo* fluorescent assay, shows clear differences between species. A distinct effect was observed following epithelium-off CXL treatment in the anterior stroma of rabbits, but no different effect was observed in horses in comparison with nontreated controls. Different protocols need to be established to effectively treat equine patients with infectious corneal disease.

**Key Words:** CXL, fluorescent biomarkers, horse, infectious keratitis, penetration depth, rabbit

## INTRODUCTION

Infectious keratitis in the form of corneal ulceration or abscessation is a serious and frequently diagnosed condition in all species treated by veterinary ophthalmologists and can lead to loss of vision or the globe in severe cases.<sup>1–4</sup> Ulcerative keratitis can progress to keratomalacia if exogenous and/or endogenous collagenolytic proteinases cause rapid degradation of the stromal collagen matrix.<sup>1,2,5</sup> Deep infectious stromal abscesses underneath an intact epithelium are more often observed in horses than in

other species. These lesions can be particularly difficult to treat.<sup>2,5,6</sup>

Ultraviolet-A (UV-A) cross-linking of corneal collagen with riboflavin as a photosensitizing agent (CXL) is a technique which is primarily used for the treatment of humans with corneal thinning disorders, such as keratoconus and ectasia after refractive laser surgery.<sup>7–9</sup> CXL has also shown promising results for the treatment of corneal infections, melting keratitis, and corneal edema.<sup>10–16</sup> Riboflavin (vitamin B<sub>2</sub>) acts as a photosensitizer when exposed to UV-A light with a wavelength at

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one of its absorption peaks (370 nm) generating free radicals, which lead to the formation of additional covalent cross-links at the surface of collagen fibrils and within the proteoglycan-rich ground substance surrounding them.<sup>17,18</sup> These cross-links increase corneal biomechanical and biochemical stability<sup>18–22</sup> through a direct increase of corneal stiffness and a reduction of enzymatic collagenolysis via steric hindrance of the collagenase-binding sites on the collagen molecule.<sup>17,22</sup> Free radicals also directly damage microorganisms and lead to apoptosis of cells in the irradiated area, and finally, riboflavin may intercalate with nucleic acids within microorganisms, inhibiting replication.<sup>23–25</sup>

Because of these biomechanical and biochemical effects, CXL appears to be a reasonable treatment modality for keratomalacia in veterinary patients. There are a few descriptions of the clinical use of CXL in veterinary ophthalmology showing promising results.<sup>26–30</sup>

Diffusion of riboflavin into the corneal stroma leading to stromal saturation is crucial for the effective absorption of UV-A radiation in the cornea and thereby determines both the efficacy and safety of CXL.<sup>18,31</sup> The standard CXL protocol includes epithelial debridement prior to riboflavin treatment (epithelium-off CXL) to optimize the diffusion of riboflavin into the stroma. However, corneal debridement causes marked discomfort and increases the risk of corneal infections in humans.<sup>32–34</sup> Veterinary patients suffering from keratomalacia usually present with large epithelial defects so that further debridement might be unnecessary, but is also unlikely to pose an additional risk.<sup>29,30,35</sup>

The standard epithelium-off CXL treatment protocol first described by Wollensak *et al.*<sup>23</sup> causes keratocyte apoptosis to a depth of approximately 300  $\mu\text{m}$  in rabbits. The depth of the treatment can actually be seen as a demarcation line using slit-lamp biomicroscopy and anterior segment optical coherence tomography.<sup>36,37</sup> Studies in rabbit, porcine, and human corneas demonstrated a consistent CXL penetration depth and showed that riboflavin shielding protects all deeper structures, including the corneal endothelium, iris, lens epithelium, and retina.<sup>31</sup> In human patients, a minimum corneal thickness of 400  $\mu\text{m}$  is recommended for CXL treatment.<sup>38–40</sup> However, little is known about the penetration depth of epithelium-off CXL in common veterinary patient species. As the equine cornea (812–835  $\mu\text{m}$ )<sup>41,42</sup> is approximately 1.5–2 times thicker than the normal rabbit (400  $\mu\text{m}$ )<sup>43</sup> and human (540  $\mu\text{m}$ )<sup>44</sup> corneas, information regarding the maximal depth at which CXL is effective would be critical for the treatment of equine patients with deep stromal abscesses. Thus, the first aim of the study was to determine the epithelium-off CXL penetration depth in equine and rabbit corneas. The rabbit data were used to compare the results of this study to previous studies evaluating CXL.<sup>17,25,45–48</sup>

The efficacy of CXL without epithelial debridement (epithelium-on CXL) is highly variable and decreased compared to epithelium-off CXL.<sup>49–52</sup> Also, the equine corneal epithelium is more than three times thicker (131–175  $\mu\text{m}$ )<sup>41,42</sup> than the normal rabbit (46  $\mu\text{m}$ )<sup>53</sup> and human (53  $\mu\text{m}$ )<sup>54</sup> corneal epithelia. Finally, infectious stromal abscesses in equine corneas typically reside underneath an intact epithelium. Thus, second aim was to evaluate the penetration depth of epithelium-on CXL in the equine cornea.

Corneal topography,<sup>55</sup> biomicroscopic examination,<sup>36</sup> *in vivo* confocal microscopy,<sup>56,57</sup> and anterior segment optical coherence tomography<sup>57</sup> are used for the assessment of the treatment effect and penetration depth of CXL in human patients. Descriptions of electron and light microscopic evaluations exist for cross-linked human<sup>58,59</sup> and rabbit<sup>23,48</sup> corneas. Immunohistochemistry has been performed in cross-linked rabbit corneas<sup>25</sup> and immunofluorescence confocal microscopy in cross-linked porcine<sup>49</sup> corneas. However, a reliable and cost-effective method to determine CXL penetration depth and the effect of CXL on corneal stromal cell integrity on a microscopic level has not yet been established. Jester *et al.*<sup>60</sup> described an assessment of keratocyte death in rabbit corneas treated with different irritating materials using fluorescent biomarkers of cell death and viability. Finally, the third aim of this study was to evaluate the use of CXL-induced cell death as an indication of CXL penetration depth in the cornea.

## MATERIALS AND METHODS

### *Treatment groups*

Rabbit and equine eyes were obtained from local abattoirs immediately after slaughter and placed in serum-free minimal essential media (MEM) (DMEM; PAA Laboratories, Pasching, Austria). Eyes were examined using a focal light source and only included if the anterior segment was normal. Eyes remained in MEM for a maximum of 4 h prior to further processing. Eyes were washed in sterile phosphate-buffered saline (PBS) (Dulbecco's PBS; PAA Laboratories) and processed according to the respective treatment group. For each species, the eyes were divided into five different treatment groups. Group 1: 17 rabbit and 10 equine eyes underwent epithelium-on CXL. Group 2: 21 rabbit and 12 equine eyes underwent standard epithelium-off CXL. Group 3: 6 rabbit and 5 equine eyes were only debrided without subsequent CXL. Group 4: 2 rabbit and 1 equine eyes without any treatment served as negative controls. Group 5: 2 eyes each were treated with topical application of 8% NaOH as positive controls of cell death.

Debridement was performed using an 8-mm-diameter sharp biopsy punch (Biopsy Punch Stiefel; GlaxoSmithKline, Muenchenbuchsee, Switzerland) to outline the area to be debrided and a microsurgical blade (Miniature Blade

no 64; Katena Products, Denville, NJ, USA) to manually remove the epithelium. Care was taken not to damage the corneal stroma. Treatment with NaOH was performed by topical application of a Schirmer tear test strip (Intervet/Schering-Plough; Animal Health, Roseland, NJ, USA) soaked with 8% NaOH on the central cornea for 20 s. Equine corneas were debrided prior to application of NaOH to allow sufficient penetration into the stroma. Rabbit corneas were not debrided as NaOH caused complete epithelial destruction as well as keratocyte cell loss throughout the stroma as determined in preliminary studies. Eyes were placed in tissue culture plates, cornea side up, with each well filled with MEM until the medium level reached the limbus.

#### CXL procedure

CXL was performed following the standard protocol as described previously.<sup>8,26</sup> Briefly, the cornea of each eye was pretreated with photosensitizing iso-osmolar 0.1% riboflavin drops (freshly mixed 0.5% aqueous riboflavin (Vit B2; Streuli, Uznach, Switzerland) and sterile 20% dextran T-500 solutions) for 30 min. Every 3 min, one drop was placed on the central cornea. A plastic ring of 8 mm or 20 mm diameter was placed on the cornea to prevent excessive fluid drain in rabbit and equine eyes, respectively. The cornea was then irradiated for 30 min with 365-nm wavelength UV-A light (irradiance: 3 mW/cm<sup>2</sup>, UV-X; Peschke Meditrade, Cham, Switzerland) focused on the corneal surface. Riboflavin solution was applied to the cornea every 3 min during the irradiation period.

#### Further processing

After the CXL procedure, all eyes were rinsed twice with PBS, followed by two rinses with MEM. The eyes were then placed in new 12-well tissue culture plates or in containers large enough to accommodate the entire equine eye. The eyes were placed cornea side up and covered with MEM. Eyes were incubated at room temperature and atmosphere for 24 h and then placed in a 10% formalin solution for overnight fixation.

After fixation, the cornea of each eye was removed and bisected. A 3-mm-wide, central strip of each hemicornea was used for the preparation of frozen and paraffin-embedded sections, respectively. Tissue for fluorescence microscopy was flash-frozen in liquid nitrogen and then stored at -20 °C overnight. Corneal strips were embedded in optimal cutting temperature compound (OCT; Biosystems, Nunningen, Switzerland) prior to section preparation. Four 7-μm-thick tissue sections of each corneal strip were collected and placed onto positively charged glass slides (SuperFrost; Biosystems) (2 sections/slide).

Slides were washed four times for 3 min with PBS and then blocked with 1% bovine serum albumin (BSA; Sigma-Aldrich, Buchs, Switzerland) in PBS for 1 h. Slides

were stained with rhodamine-labeled phalloidin (R415 rhodamine phalloidin; Life Technologies, Zug, Switzerland), using three units per section, diluted 1:2 in PBS to label cells unaffected by the CXL procedure, and counterstained with 4',6-diamidino-2-phenylindole (DAPI) at a 1:1000 dilution in PBS (DAPI 300 nM; Life Technologies) to label cell nuclei. Staining was performed for 2 h. All sections were washed four times for 3 min with PBS and then cover-slipped using mounting medium (Fluoromount FP-483331; Interchim, Montluçon, France).

The paraffin-embedded second hemicornea was routinely sectioned and stained with hematoxylin and eosin (HE).

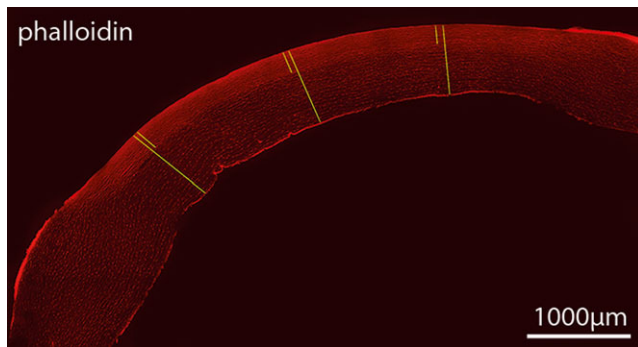
#### Evaluation

Fluorescent sections were evaluated at 10× magnification with an epifluorescence microscope equipped with appropriate filters (Leica DM 6000B; Leica Microsystems, Heerbrugg, Switzerland). To acquire an adequate image of each whole corneal section, a number of single images were acquired and merged into one composite image using a microscope compatible camera (Leica DFC 350 FX; Leica Microsystems, Mannheim, Germany) and specific merging software (Leica Microsystems). Fluorescent images were viewed and analyzed using IMAGEJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD, USA; available at <http://rsb.info.nih.gov/ij/index.html>).

Light microscopic images were scanned (NanoZoomer 2.0; Hamamatsu, Solothurn, Switzerland), viewed, and analyzed using special software (NDP.view2; Hamamatsu).

Two different observers (NG and SP) independently evaluated 2–4 sections of each cornea quantitatively and qualitatively. Qualitative evaluations were performed on all three different stains. On phalloidin-stained slides, actin cytoskeleton staining and collagen compaction were evaluated. The absence of actin cytoskeleton staining indicates cellular disintegration and, therefore, keratocyte death,<sup>60</sup> which was interpreted as immediate visible effect indicating penetration depth of the CXL treatment. DAPI-stained slides were used for the evaluation of keratocyte nucleus morphology. HE-stained slides were used for evaluation of keratocyte nucleus morphology and collagen compaction. Pyknotic nuclei are signs of cell death and were also considered as visible effect indicating penetration depth of the CXL treatment. Quantitative evaluations were only performed on phalloidin-stained specimens. Measurements of actin cytoskeleton staining loss, which was interpreted as the penetration depth of the CXL procedure, were performed at three different locations in each corneal section as previously described by Jester *et al.*<sup>60</sup> and as shown in Fig. 1: The entire stromal thickness and the depth of actin cytoskeleton staining loss were measured. The latter distance was reported in μm and as a percentage of total stromal thickness.





**Figure 1.** Phalloidin-stained rabbit cornea that underwent epithelium-off CXL. The yellow lines indicate the manner in which measurements were performed. The entire stromal thickness and the depth of visible actin cytoskeleton staining loss, which coincides with the area in which the collagen lamellae are compacted, were measured at three different locations in each corneal section as previously described by Jester *et al.*<sup>60</sup>

### Statistical analysis

Statistical analysis was performed using the programs lme4 and nlme of R statistical software (www.r-project.org). A linear mixed effects model was used to assess differences between groups and between observers. To avoid potential clustering within eyes, these were treated as random effect. Model selection was based on Akaike information criterion (AIC) with lower values indicating a better model fit. Statistical significance was defined as  $P < 0.05$ .

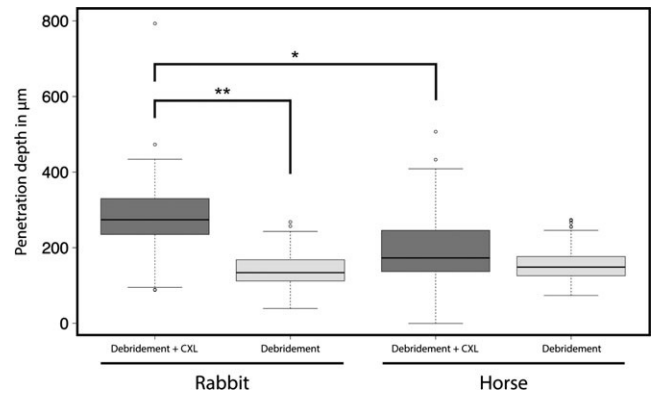
## RESULTS

The qualitative results are supported by the quantitative evaluation. The results of the quantitative evaluation are shown in Fig. 2.

No statistically significant differences in measurements were registered between the two observers.

### Epithelium-off CXL penetration depth in rabbit and equine corneas

Rabbit eyes that underwent standard epithelium-off CXL (group 2) demonstrated visible effects in the anterior one-third to half of the corneal stroma. On phalloidin-stained slides, actin cytoskeleton staining was absent in the anterior one-third to half of the corneal stroma. This area of actin cytoskeleton staining loss overlapped with an area of anterior stromal collagen compaction (Fig. 3d). On DAPI-stained slides, keratocyte nuclei appeared smaller and more fluorescent in the anterior one-third to half of the stroma (Figs 3e and f). Arrows in Fig. 3f indicate pyknotic nuclei and arrowheads indicate nucleus fragments. The zone of pyknotic nuclei overlapped with the zone of actin cytoskeleton staining loss and collagen compaction. On HE-stained slides, keratocyte nuclei appeared pyknotic in the anterior one-third and there was collagen compaction in the anterior half to two-thirds of

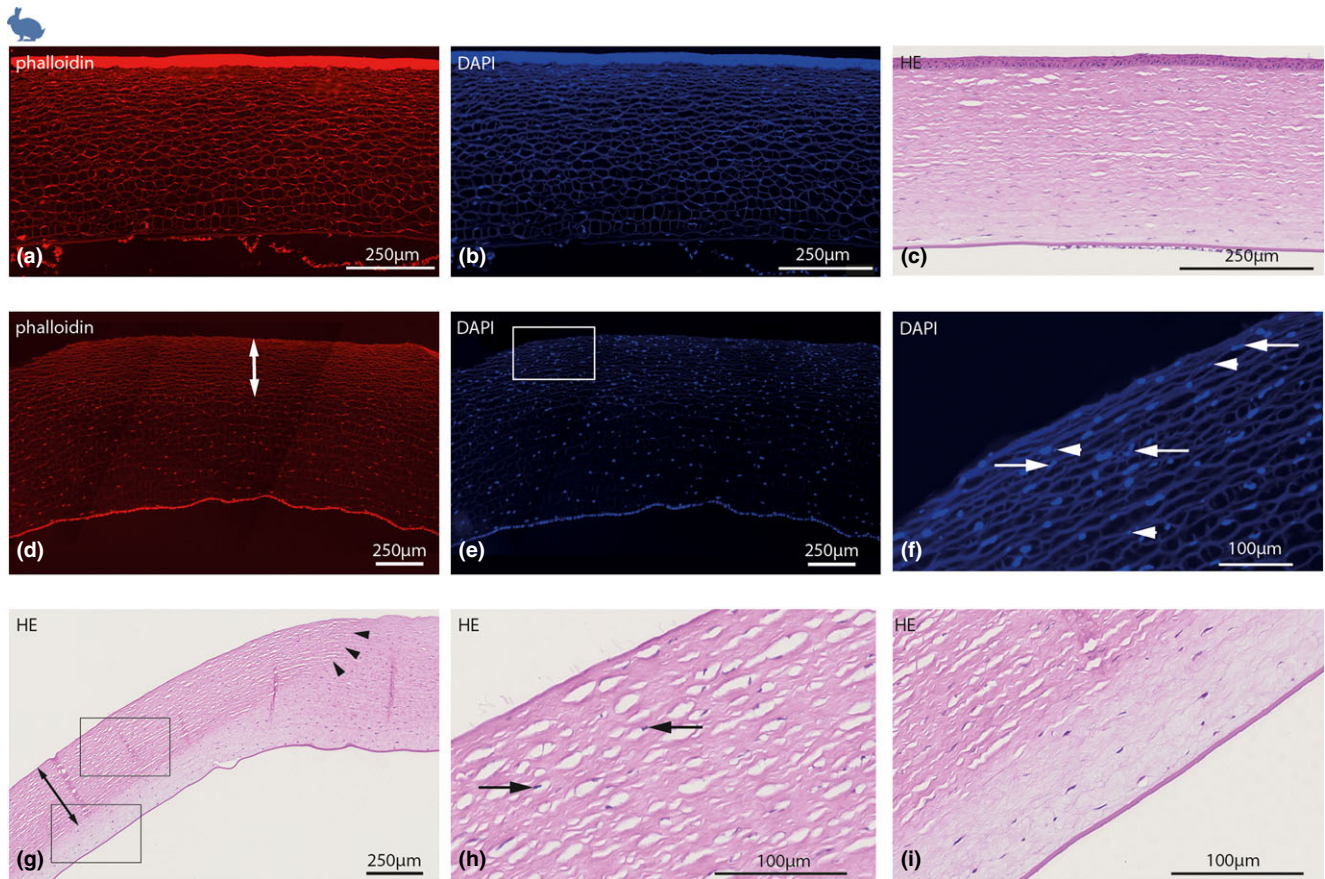


**Figure 2.** Boxplot image comparing the median penetration depth in  $\mu\text{m}$  in rabbit corneas (left) and equine corneas (right). Corneas were subjected to debridement and CXL (experimental group 2: epithelium-off CXL) and debridement only (experimental group 3). In rabbits, the absolute actin cytoskeleton staining loss was significantly deeper in corneas that underwent debridement and CXL than in corneas that were debrided only (\*\* $P < 0.001$ ). In horses, no statistically significant difference was observed between the two groups. The median penetration depth in absolute numbers was statistically significantly deeper in rabbit than in equine corneas that underwent debridement and CXL (\* $P < 0.01$ ).

the stroma (Figs 3g and h). A sharp delineation between untreated peripheral and treated central stroma was visible (Fig. 3g). These effects were significantly different from the effects observed in the positive and negative control groups (groups 3, 4 and 5).

Rabbit corneas that underwent standard epithelium-off CXL (group 2) showed a median penetration depth of 274  $\mu\text{m}$  (interquartile range IQR: 235–330  $\mu\text{m}$ ). This corresponded to 32% (IQR: 28–38%) of the total stromal thickness. Rabbit corneas that were only debrided without subsequent CXL (group 3) showed a median actin cytoskeleton staining loss of 134  $\mu\text{m}$  (IQR: 112–168  $\mu\text{m}$ ) or 13% (IQR: 11–15%) of the total stromal thickness. The actin loss in rabbit corneas that underwent epithelium-off CXL was statistically significantly larger than the actin loss of rabbit corneas undergoing debridement alone ( $P < 0.001$ ) (Fig. 2).

Equine corneas that underwent epithelium-off CXL (group 2) also demonstrated actin cytoskeleton staining loss and collagen compaction in the phalloidin-stained slides. However, this effect was restricted to the most superficial anterior stroma, comprising one tenth or less of the total stromal thickness (Figs 4d and e). On DAPI-stained slides, pyknotic nuclei overlapped with the zone of superficial actin cytoskeleton staining loss (data not shown). On HE-stained slides, pyknotic nuclei were visible in the most superficial anterior stroma, but no collagen compaction could be appreciated (Fig. 4f). Apart from the collagen compaction observed in the phalloidin-stained slides, these effects were identical to the effects observed in the negative control group (both rabbit and equine corneas) receiving debridement alone without subsequent CXL (group 3).



**Figure 3.** The penetration depth of CXL in rabbit corneas in the presence or absence of the corneal epithelium. (a–c) Epithelium-on CXL, note normal appearance of cornea. (d–i) Epithelium-off CXL. (d) Actin cytoskeleton staining loss and collagen compaction, outlined by the white arrow, were visible in the anterior third of the stroma. Swelling of the posterior stroma and an increased total corneal thickness can be observed. (e) Cell nuclei in the anterior third of the stroma were smaller and more fluorescent. (f) Magnification of (e). Pyknotic nuclei and nucleus fragments are indicated by the white arrows and arrowheads, respectively. (g) Collagen compaction is present in the anterior two-thirds of the stroma and is indicated by the black arrow. The arrowheads outline the sharp delineation between untreated peripheral and treated central cornea. (h) Magnification of anterior stroma of (g). Arrows indicate pyknotic nuclei in the anterior third of the stroma. (i) Magnification of posterior stroma of (g). Keratocyte nuclei are larger and more regularly shaped than in the anterior stroma.

Equine corneas that underwent epithelium-off CXL (group 2) showed a median penetration depth of 173  $\mu\text{m}$  (IQR: 137–246  $\mu\text{m}$ ) or 9% (IQR: 6–14%) of the total stromal thickness. The penetration depth of the epithelium-off CXL procedure in  $\mu\text{m}$  was statistically significantly less in equine corneas compared to rabbit corneas ( $P < 0.01$ ) (Fig. 2).

Equine corneas that were only debrided without subsequent CXL (group 3) showed a median actin cytoskeleton staining loss of 149  $\mu\text{m}$  (IQR: 126–177  $\mu\text{m}$ ) or 8% (IQR: 7–10%) of the total stromal thickness. This did not statistically significantly differ from the median actin cytoskeleton staining loss of equine corneas undergoing epithelium-off CXL ( $P = 0.401$ ) (Fig. 2).

#### *Epithelium-on CXL penetration depth in equine corneas*

Rabbit and equine eyes that underwent epithelium-on CXL (group 1) were similar to untreated negative controls (group 4) for all stains (Figs 3a–c and 4a–c): Collagen

lamellae were evenly spaced with mildly higher packing density in the anterior stroma when compared to the posterior stroma. Cell nuclei were of regular dimensions and were evenly spaced throughout the stroma.

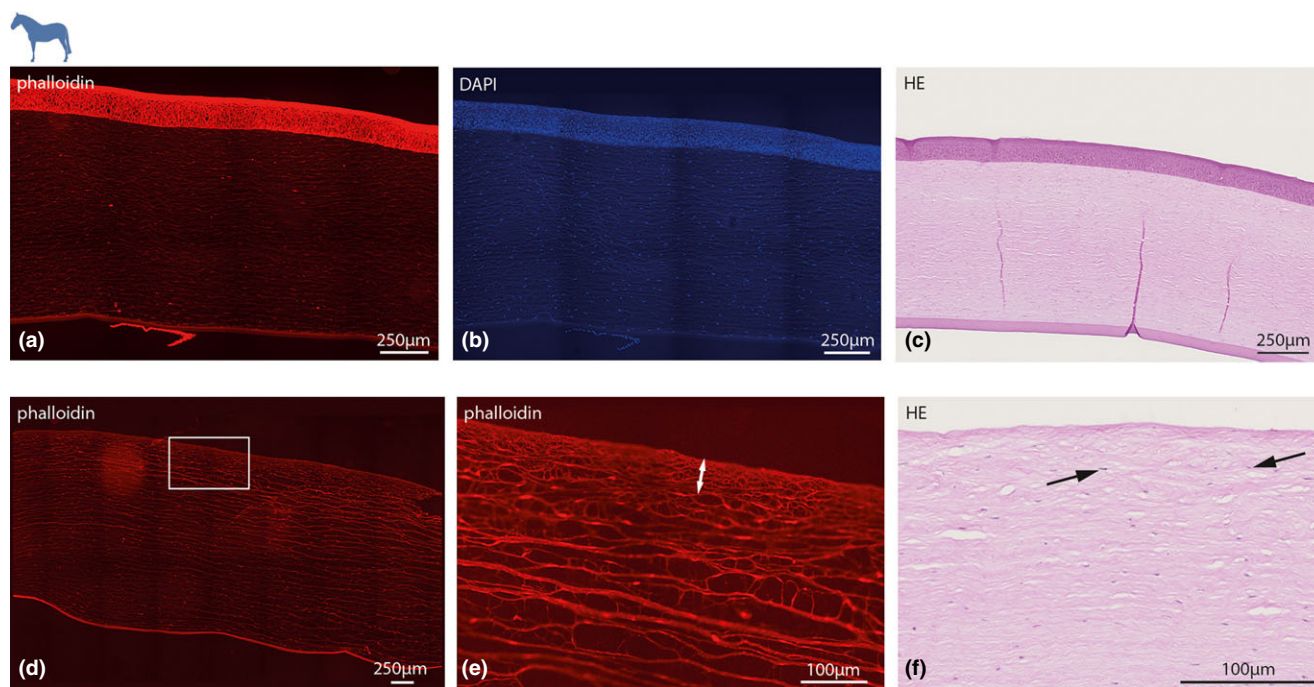
Rabbit and equine corneas that underwent epithelium-on CXL (group 1) and untreated rabbit and equine corneas (group 4) did not show any measurable effect: penetration depth 0% (data not shown).

Rabbit and equine eyes treated with topical application of NaOH (group 5) showed complete cell loss in all layers of the cornea underneath the treated area in DAPI-, phalloidin-, and HE-stained specimens and thus a penetration depth of 100% (data not shown).

#### *Evaluation of the use of CXL-induced cell death as an indication of CXL penetration depth in the cornea*

The results observed via fluorescent (phalloidin and DAPI) and conventional light microscopy demonstrated good correlation within this study and with published data.





**Figure 4.** The penetration depth of CXL in equine corneas in the presence or absence of the corneal epithelium. (a–c) Epithelium-on CXL, note normal appearance of cornea. (d–f) Epithelium-off CXL. (d) Actin cytoskeleton staining loss and collagen compaction at the anterior margin of the stroma. (e) Magnification of (d). The white arrow outlines the actin cytoskeleton loss and collagen compaction. (f) Pyknotic nuclei are visible in the anterior margin of the stroma and are outlined by black arrows.

## DISCUSSION

The present study demonstrated that fluorescent biomarkers could be used to reliably determine CXL-induced stromal cell loss, which was interpreted as a measure of immediate CXL treatment effect and penetration depth. Stromal collagen compaction and changes in keratocyte nucleus morphology as a result of epithelium-off CXL in the anterior 300  $\mu\text{m}$  of stroma have been described previously and were observed to overlap with the zone of actin loss in epithelium-off CXL-treated corneas in the present study.<sup>23,25,49</sup> The epithelium-off CXL penetration depth observed in rabbits in the present study (median 274  $\mu\text{m}$ ) corresponded well with the published literature.<sup>23,61</sup> The effect of epithelium-off CXL in equine corneas was evaluated for the first time and epithelium-off CXL demonstrated an unexpectedly shallow effect in the equine cornea (median 173  $\mu\text{m}$ ). Interestingly, the demarcated epithelium-off CXL penetration depth in absolute numbers was significantly larger in rabbit compared to equine corneas. Loss of actin cytoskeleton staining, as an indicator of keratocyte death,<sup>60</sup> is a known effect observed after debridement alone<sup>23,62–64</sup> and was observed in the present study. Specifically, rabbit and equine corneas that were just debrided demonstrated actin cytoskeleton staining loss and pyknotic nuclei, but these effects were only visualized in the most anterior stroma, and collagen

compaction was not observed. Actin loss was observed to a significantly greater depth in the stroma of rabbit corneas treated with epithelium-off CXL compared to corneas that were only debrided.<sup>47</sup> The penetration depth in equine corneas that underwent epithelium-off CXL was superficial and could not be distinguished from the effect of debridement alone.

The effect of epithelial debridement prior to the CXL procedure on CXL penetration depth was profound in the present study. No effect could be observed in rabbit and horse eyes undergoing epithelium-on CXL. This result also corresponds to a recently published study in humans showing no effect of epithelium-on CXL in progressive keratoconus with a follow-up of 2 years.<sup>50</sup> The tight junctions of the corneal epithelium act as a major permeability barrier to riboflavin/dextran solutions, and debridement is therefore regarded as a prerequisite for the standard CXL protocol.<sup>8,40,49–52,65</sup> On the other hand, epithelial debridement carries the disadvantages of discomfort and greater risk for infections in humans.<sup>32,34</sup> For these reasons, several studies have evaluated alternatives to achieve sufficient stromal riboflavin concentrations without debridement, using a variety of methods.<sup>32,34,65–69</sup>

Rosetta *et al.*<sup>35</sup> and Famose *et al.*<sup>29,30</sup> described the application of CXL with a window application for infectious ulcerative keratitis, whereby riboflavin penetration into the stroma is obtained through the epithelial defect

overlying the ulcer without or with minimal further debridement. This window application demonstrates that corneas with an intact epithelium may only need debridement of a small area of epithelium to allow for sufficient riboflavin diffusion.

The penetration depth has not been studied in horses before and the question now arises how effective CXL (epithelium-on and epithelium-off) in this species would be clinically, especially for deep stromal pathologies and in view of a greater stromal thickness in horses when compared to other species. A recent clinical case series described CXL treatment in addition to medical therapy in nine horses with ulcerative keratitis.<sup>28</sup> The authors applied the standard CXL protocol and judged postoperative scarring as impressively mild and the healing time as rather fast in eight of nine horses. One horse with fungal keratitis was enucleated at 4 days after treatment due to panophthalmitis. Because of the low number of patients and the absence of a control group in the study, the authors concluded that CXL might potentially be useful for the treatment of stromal ulcers in horses.<sup>28,70</sup> However, the results of the present study suggest that efficacy would not be expected with epithelium-on or off CXL in horses with infectious keratitis because CXL was unable to penetrate beyond the superficial normal equine cornea. Further studies are required to determine CXL penetration depth in infected equine corneas with or without keratomalacia. The current CXL protocols need modification to modulate CXL penetration depth and thus fit various species and treatment indications. A tailored increase in total radiation energy delivery might increase CXL penetration depth in the thick equine cornea and also increase the antimicrobial effect without elevating the risk for endothelial damage.

The detection method for CXL penetration (assessment of cell death and collagen compaction) was a possible limitation of the present study. An inconsistent correlation between the degree of keratocyte apoptosis and corneal mechanical stiffening has been observed in a mouse model **8** for CXL (Kling S. *et al.*, personal communication). This suggests that the apoptotic effects of CXL do not necessarily correlate with the depth or degree to which the physicochemical CXL effects occur in the cornea. These effects would not have been observed in the corneas used in this study, because only cytotoxic effects were evaluated.

Significant differences in riboflavin-mediated UV-A absorption rates were not observed between human, porcine, ~~bovine~~, and equine corneas in a recent study **9** (Wuarin R. *et al.*, personal communication). The authors concluded that the known riboflavin diffusion rates for humans can be used without modification and that a species-specific customization of CXL in terms of riboflavin saturation should not be necessary. However, riboflavin-mediated UV-A absorption is only one of

several elements influencing the effectiveness of CXL: Interspecies variations in ~~ECM~~ and proteoglycan composition<sup>71</sup> may affect covalent bond formation in the corneal stroma.<sup>72</sup> Energy delivery may thus need to be adapted to specific species, independently of species-specific corneal thickness. Other possible reasons for the shallower zone of CXL penetration and keratocyte loss in horses could be differences in oxygen availability or keratocyte susceptibility to free radical-induced damage.<sup>73</sup>

Assessment of the relative penetration depth of CXL presented a challenge in the present study, as corneas incubated in medium for 24 h inevitably show stromal swelling that primarily occurs in the posterior stroma.<sup>74</sup> A reduced swelling capacity of the anterior cross-linked stroma has been described<sup>75</sup>; therefore, assessment of the absolute penetration depth seems reasonable, but the relation of the anterior cross-linked stroma to the total stromal thickness might be erroneous. In the present study, different posterior stromal swelling capacities were determined in cryopreserved sections compared to paraffin-embedded sections. This observation is probably caused by dehydration, clearing, and paraffin wax embedding of the HE sections, which did not affect the cryopreserved sections, used for the fluorescence images.<sup>76,77</sup>

The authors think that the anterior cross-linked to total stromal thickness ratio of 2/3 observed in the paraffin-embedded sections (Fig. 3g) might be more representative of the true effect than the anterior cross-linked to total stromal thickness ratio of 1/3 observed in the cryopreserved sections. This assumption is based on the following calculation: If the cross-linked anterior two-thirds of the stroma in rabbits correlates with 274  $\mu\text{m}$  (the median CXL penetration depth observed in this species), the total stromal thickness of the same cornea is approximately  $274 + 137 = 411 \mu\text{m}$ , which corresponds to the published corneal thickness in rabbits.<sup>43</sup>

In conclusion, the present study shows that the absolute penetration depth of CXL can be determined using fluorescent biomarkers. The current CXL protocols need modification to fit various species and treatment indications, as demonstrated in the equine corneas in this study. In addition to transepithelial riboflavin permeability, future studies should focus on the modulation of CXL penetration depth in horses.

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